An approach to three-dimensional analysis of cambial cells and their derivatives in Robinia pseudoacacia L

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ハリエンジュの形成層とその派生細胞の3次元的解析への試み

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Résumé

For the three-dimensional analysis of cambial cells and the derivatives in hardwoods, *Robinia pseudoacacia* having the storied cambium was offered to the serial sectioning from the transverse surface, and the cell structures were reconstructed from the series. Several microscopic improvements were attempted for the precise analysis. As the result, any peculiar cell corresponding to the cambium initial was not found out. Wood fibers were shown not to expand transversely but to elongate longitudinally by the tip growth during the differentiation, being quite different from the tracheid in softwood.

要　旨

組織が複雑で，細胞寸法も小さい広葉樹の形成層とその派生細胞については，針葉樹に比べて観察例が非常に小ない。そこで広葉樹の中でも進化した木部を形成すると言われているハリエンジュを供試材として，光顕法と電顕法を組み合せた観察や，2 μm 厚さの50～1000枚の連続木口切片を再構築することにより，主として紡垂形形成層細胞と木繊維の発達過程を調べた。その結果，始原細胞に相当する細胞は認められず，形成層帯の細胞は一様であった。木繊維への分化においては，細胞の形状が主として水平方向に拡大する針葉樹仮道管の場合とは異なり，ハリエンジュでは偏平な四角形の断面形状を持つ形成層細胞が多角形へと形状を変えてはいるが，横断面の周囲長自体には変化が認められなかった。これに対して細胞長軸方向へは，形成層細胞の5倍にも伸長し，その伸長は主として細胞の両端部で進行すると判断された。

Introduction

The cambial activity and the differentiation of the derivatives have been studied mainly in softwoods for a long time, because the size of softwood derivatives, expecially of tracheids, is proper to be examined by LM (light microscopy), and the tracheids are produced constantly from the cambial cells in spring. Also they elongate little during their differentiation, so the sequence of their developments can be traced easily on a single transverse section. The present authors have also reported several papers about the cell wall formation of some softwoods

from the viewpoints of cytology and chemistry.[1–4]

On the contrary, the cambial cells and their derivatives are very small in hardwoods. The derivatives elongate remarkably in the fiber development, and expand enormously in the vessel-development. The precise analysis on hardwood formation, therefore, are very difficult and the previous studies are few in comparison with those of softwoods.[5–7]

For the precise examination of smaller and complicated hardwood tissues, higher resolution of LM and also three-dimensional approach must be introduced to the observation. In this paper HARIENJU (*Robinia pseudoacacia*) was used and the fiber development was traced mainly along the cell axis. Some microscopic techniques were improved for the three-dimensional analysis of the cell structure.

Materials and Methods

On May 18th, 1982 and on June 3rd, 1983, fresh specimens were taken from trunks of *Robinia pseudoacacia* L growing on the campus of Kyoto University and having about 100 cm D. B. H. They were soaked immediately into 3% GA (glutaraldehyde) and subdivided into smaller blocks of $5\times2\times3$ (L×T×R) mm^3 after 3 hrs. They were fixed again by 1% OsO_4 (osmium tetroxide) or the combination of 3% TA (tannic acid) and 1% OsO_4, and embedded in epoxy resin[8]. These blocks were treated by the ordinary preparation techniques of ultrathin sectioning for TEM (transmission electron microscopy) and of semithin sectioning for LM.

Results and Discussion

Microscopy When the effects of fixation and embedding were checked, cell structures were preserved well and also the contrast of cytoplasm and unlignified cell wall were enhanced in the GA-TA-OsO_4 fixed specimen by the osmium black. The contrast could be traced without any other staining for LM, although it was a little coarse for TEM. Several staining were attempted on semithin sections of 2 μm thick which were adhered on the slide only by drying[8].

Safranin which had been shown to be very effective to the lignified cell wall[8] gave good contrast by the combination with GA-TA-OsO_4 fixation. The staining procedure was simplified, namely, 0.01% aqueous solution was dropped on the sections and washed only by water.

Methylene blue[9] stained cytoplasm, especially nuclei (Fig. 1B), although the discoloration was soon.

Basic fuchsin[9] and *toluidine blue* were not so specific as above two stainings.

Acid Shiff and *silver-methenamine*[10] after the periodation were effective to cell walls and starch grains. Especially, the contrast originated from silver was very dense, and useful to transport directly the figures from LM to the texture analyzing system, when the staining period was prolonged to get more dense contrast.

Critical correspondence between LM and TEM A transverse surface of the specimen fixed by GA-OsO_4 was trimmed to 0.5×0.4 mm^2, and set on a LKB ultramicrotome. About 10 sheets of ultrathin sections under 0.1 μm thick were sliced by a glass knife and picked

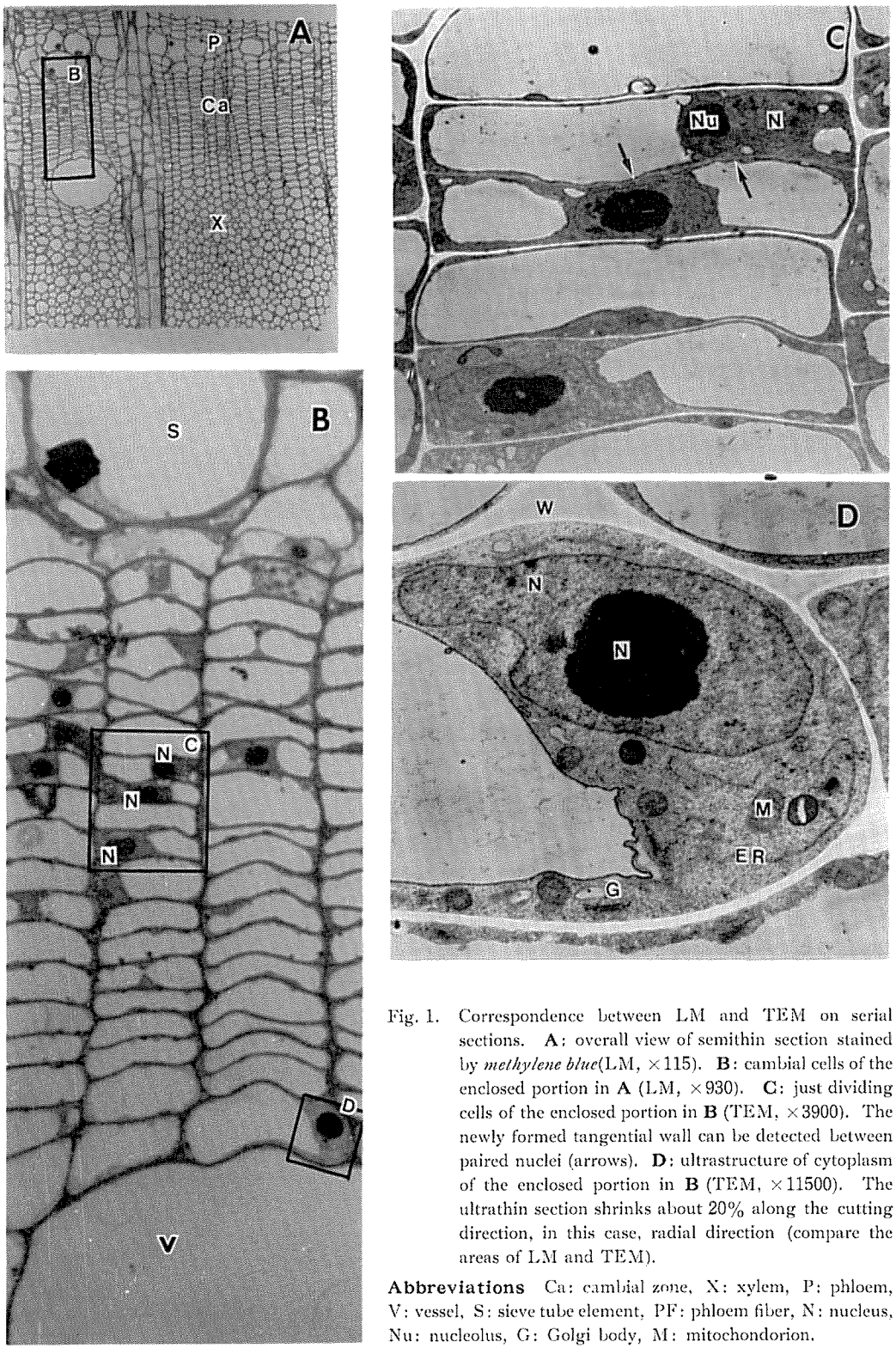

Fig. 1. Correspondence between LM and TEM on serial sections. **A**: overall view of semithin section stained by *methylene blue*(LM, ×115). **B**: cambial cells of the enclosed portion in **A** (LM, ×930). **C**: just dividing cells of the enclosed portion in **B** (TEM, ×3900). The newly formed tangential wall can be detected between paired nuclei (arrows). **D**: ultrastructure of cytoplasm of the enclosed portion in **B** (TEM, ×11500). The ultrathin section shrinks about 20% along the cutting direction, in this case, radial direction (compare the areas of LM and TEM).

Abbreviations Ca: cambial zone, X: xylem, P: phloem, V: vessel, S: sieve tube element, PF: phloem fiber, N: nucleus, Nu: nucleolus, G: Golgi body, M: mitochondorion.

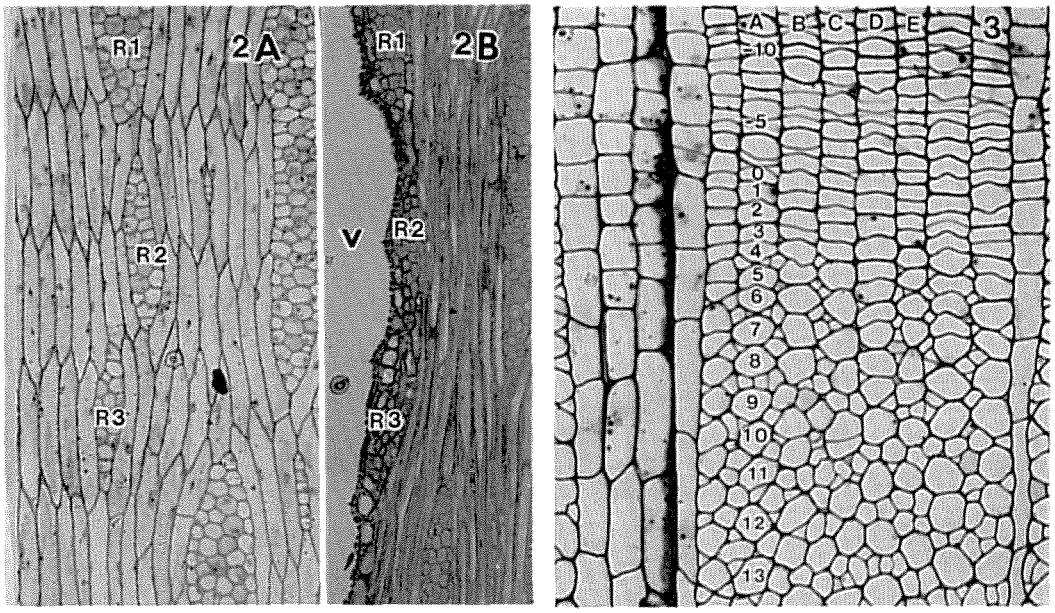

Fig. 2. Pair of serial tangential sections on cambial zone (**A**) and on differentiating zone (**B**) (LM, ×115). Fusiform cambial cells show stories and have a long "body" and two short "tips" in **A**. Differentiating fibers are very complicated in **B**, although rays can be traced through serial sections (see symbols).

Fig. 3. The 450th section contained in 800 serial sheets of 2 μm thick (LM, ×320). Cells are identified by cell number as shown in radial file **A**.

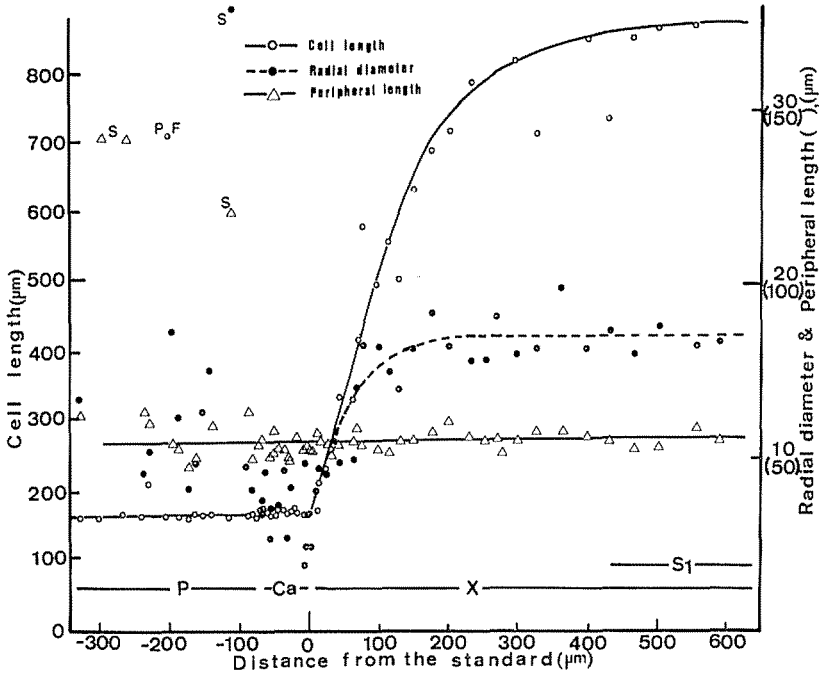

Fig. 4. Cell lengths, radial diameters and peripheral lengths in radial file **A** (cf. Fig. 3). The elongation of cell is very rapid at the beginning of fiber differentiation. The peripheral lengths are constant on cambial cells and differentiating wood fibers, in spite of the increment of radial diameter.

on a grid. Succeedingly, 3 sheets of 2 μm sections were got at the same knife position and put on a glass slide. The procedures were repeated at the neighboring new knife position. These sets of ultrathin and semithin sections were observed by TEM and LM after stainings, respectively (Fig. 1). Wide area was surveyed under low magnification of LM (Fig. 1A). and the ultrastructure on cell walls and cytoplasms were examined by TEM (Fig. 1C, D). Semithin sections also enabled the observation of cell structures under the high resolution of LM (Fig. 1B). From the comparison of the dimension between the sections and the block surface after sectionings, it was found out that the ultrathin sections shrinked seriously along the cutting direction, while the shrinkage of the semithin sections was negligible (cf. Figs. 1B and 1C). As described above, TEM and LM have merits and demerits with each other, so the correspondence between these two methods is considered to be very important for the precise examination of fine tissue such as that of hardwood.

In this study by the combination of them, any peculiar cell in the cytological aspect could not be found out, corresponding to the cambium initial.

Three-dimensional reconstrution of serial sections Transverse textures of the cambial cells and their derivatives have been studied by many investigators, because the texture can be observed immediately on a single transverse section. However, the cambial activity and the differentiation of the derivatives should be examined more precisely from the three-dimensional point of view. Serial tangential sections of some softwoods were used for the analyses of rise and fall of radial files by Bannan[11] and others[12]. Although the same method was applied to this study, it was impossible to trace the radial files, to say nothing of individual cells, because of extreme reorientation of the xylem cells (Fig. 2).

Therefore, cell structures were reconstructed from the serial transverse sections, even though the procedure is very troublesome. As shown above, 2 μm semithin sections were effective for the precise examination of hardwood tissues by LM. So serial sheets were sliced from the specimens fixed by GA-TA-OsO_4. For the exact reconstruction of serial sections some geometrical standards are necessary through them and also the sectioning interval must be measured. The specimen blocks were elaborated to have a flat surface on both tangential- and radial-sides by the similar technique to that of Takabe et al.[3] Then, 500–1000 sheets were sliced from a transverse surface by glass knives and a JB-4 microtome of which the thickness indicator was set on 2 μm. The nominal thickness was revised by the sectioning of a photographic film on which a microscale had been taken. As the result, the indicator was shown to be correct. About every 50 times of sectioning the cutting knife-edge was moved to new position, and the loss during the readjustment at the new position was estimated to one-sheet thick.

The serial sheets were distributed alternately on five sets of glass slides. *Safranin, basic fuchsin-methylene blue* and *silver-methenamine* were applied to each set and others were reserved. The cell structures contained in the block were reconstructed from the series of picture on photographic papers. Two flat surfaces elaborated before serial sectioning were expected to be used as the standards for the precise three-dimentional reconstruction of serial ones. However, these standard surfaces were sometimes distorted a little by the affection of cutting. Therefore, fusiform cambial cells and their derivatives were traced only vertically. Since any paticular cell such as the cambium imtial could not detected in a radial file, the

innermost cambial cell, which was just before cell from the rapid elongation of wood fiber (cf. Fig. 5), was used as the standard cell in each radial file and numbered 0 (Fig. 3). Then, positive and negative numbers were given to xylem side and also cambial side, respectively (Fig. 3).

First of all, cell lengths were calculated from the both section numbers at the upper tip and at the lower tip in several radial files which passed through far or near vessels, or contained vessel elements. One case of radial file A in Fig. 3 which passes through far from vessels is shown in Fig. 4. The lengths are constant (about 180 μm) at the cambial zone, and elongate very rapidly at the early stage of wood fiber differentiation. The elongation was up to about 5 times in wood fibers and 4 times in phloem fibers. When the radial file passed through near vessels or contained vessels, the elongation of fibers was not so remarkable at the peripheral position of the vessels, and the vessel elements themselves did not elongate at all, of course.

Second, radial diameters and peripheral lengths were measured at the center of cell and traced in several radial files (Fig. 4). The diameter are varying at the cambial cells on account of their repeated periclinal division. The diameter of wood fibers did not so increase as their length and soon leveled off. On the contrary, it is very interesting to note that the peripheral lengths were constant all over the cambial cells and the differentiating fibers in spite of the considerable increase in their radial diameter. This implies that the transverse shape of wood fibers changes from the flat rectangular at the cambial zone to the polygon (Figs. 1B and 3), being not followed by any net extension of the wall, which is remarkable in the tracheid differentiation of softwoods.

The cell extension of wood fibers in *Robinia pseudoacacia* can be characterized by the longitudinal elongation. *Robinia* is generally considered to belong to the most evolved species in hardwoods[7], so this character seems to suggest the course of evolution from the tracheids, which have the bifunction of water conduction and mechanics, to the fibers having only the mechanical function. If more species of hardwoods are examined, this course may be shown clearly.

Third, the position of nucleus was traced along the cell axis. In the cambial cells nuclei were specifically located at the center of cells. They maintained their location during the fiber differentiation at the central position in spite of the enormous elongation of fibers. This suggests that the cell wall near a nucleus is not extended vertically but the extension is proceeded mainly on the wall at tip regions. When the cell shapes were observed on the tangential section at the cambial zone (Fig. 2A), the fusiform cells were shown to have a central long "body" and two short "tips". The peripheral length reflecting the cross sectional shape of a cell was measured and traced along the cell axis, because the shape itself is difficult to be evaluated. The results in the radial file A

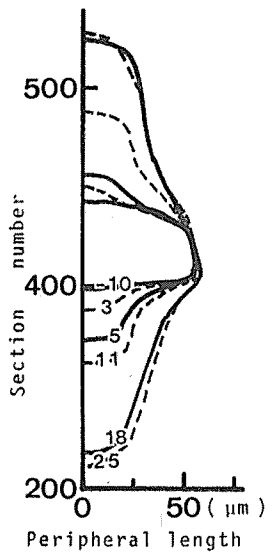

Fig. 5. Peripheral lengths along cell axis of cambial cell (−10), and of the differentiating cells (3, 5, 11, 18and 25) in radial file A in Fig. 3.

were accumulated and overlapped with each other on Fig. 5. It is interesting to note that the "full-cheeked" character of "body" of cambial cell (—10) is persistent during the differentiation of wood fibers, while "tips" seem to elongate. Being judged from these evidences, the rapid elongation of fibers is proceeded dominantly by the tip growth.

In this study the three-dimensional approach and the correspondence between LM and TEM were performed on the separate specimen blocks. If these techniques are jointed together, it is expected that more informations can be obtained.

References

1) Fujita, M., Saiki, H. and Harada, H.: The secondary wall formation of compression wood, III. *Mokuzai Gakkaishi.* **24**: 366–361, 1978
2) Fujita, M. and Harada, H.: Autoradiographic investigation of cell wall development, II. *ibid.*. **25**: 89–94, 1979
3) Takabe, K., Fujita, M., Saiki, H. and Harada, H.: Changes in the composition and the absolute amount of sugars with the development of Cryptomeria tracheids. *ibid.*. **29**: 183–189, 1983
4) Takabe, K., Fujita, M., Saiki, H. and Harada, H.: Incorporation of the label from ^{14}C-glucose into cell-wall components during the maturation of Cryptomeria tracheids. *ibid.*. **30**: 103–109, 1984
5) Wakuta, R., Saiki, H. and Harada, H.: Enlarging of differentiating vessel element in *Firmiana platanifolia* Shott et. Endl. *Bull. Kyoto Univ. Forests.* **45**: 204–216, 1973
6) Catesson, A. M. and Roland, J. C.: Sequential changes associated with cell wall formation and fusion in the vascular cambium. *IAWA Bull.*. **2**: 151–162, 1981
7) Philipson, W. R., Ward, J. M. and Butterfield, B. G.: "The vascular cambium." Chapman & Hall LTD, London, 1971
8) Fujita, M., Saiki, H., Sakamoto, J., Araki, N. and Harada, H.: The secondary wall formation of compression wood tracheid, IV. *Bull. Kyoto Univ. Forests.* **51**: 247–256, 1979
9) Huber, J. D., Rarey, F. and Odland, G. F.: Basic fuchsin and methylene blue rapid stain for epoxy-embedded tissue. *Stain Technol.*. **43**: 83–87, 1968
10) Roland, J. C.: General preparation and staining of thin sections. in "Electron microscopy and cytochemistry (J. L. Hall, ed.)", Elsevier/North-Holland Biomedical Press, Amsterdam, p. 1–43, 1978
11) Bannan, M. W.: The frequency of anticlinal division in fusiform cambial cells of Chamaecyparis. *Amer. J. Bot.*. **37**: 511–519, 1950
12) Cumbie, B. G.: Developmental changes in the vascular cambium of *Leitneria floridana*. *Amer. J. Bot.*. **54**: 414–424, 1967